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INFLUENCE OF BDNF GENOTYPE AND EXERCISE ON BDNF SERUM LEVELS AND VO_2 MAX AFTER ACUTE EXERCISE AND POST-TRAINING

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14. ABSTRACT Exercise has been associated with improvements in attention, memory and metabolic capacity (VO_2Max). Differential effects of exercise type on these variables have been seen. A large body of evidence suggests better VO_2Max is associated with better cognitive function. Brain-derived neurotrophic factor (BDNF) which exists as two main polymorphisms (alleles), has been shown to effect cognitive capacity via exercise stimuli, and modulates response to exercise. This retrospective cohort study investigated BDNF allele association with exercise type. Outcome variables were VO_2Max and serum BDNF levels. Preliminary results suggest that BDNF allele type does affect post-training VO_2Max and post-acute exercise bout BDNF serum level regardless of high (HC) or low cognitive exercise training type. Also, two different exercise types produced different VO_2Max and post-acute exercise BDNF serum levels regardless of allele. Each allele type has been associated with variable effects on memory and attention. Further study is necessary to fully characterize BDNF allele effects by exercise type on Airman cognitive and physiological outcomes to facilitate precision exercise prescription and training.

15. SUBJECT TERMS cognitive, physical training, BDNF, Val66Val, Val66Met, VO_2Max

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PREFACE

Funding for the Post Testing molecular research was provided through the Aerospace Toxicology Program, which is part of the Aerospace Physiology and Toxicology Program in the 711 Human Performance Wing of the Air Force Research Laboratory. Part of the molecular research was conducted under cooperative agreements FA8650-10-2-6062 and FA8650-15-2-6608, both with the Henry M. Jackson Foundation for the Advancement of Military Medicine (HJF). The program manager for the HJF cooperative agreements was David R. Mattie, PhD (711 HPW/RHDJ). The technical manager for the post testing was Camilla A. Mauzy (711 HPW/RHDJ).

The use of human blood samples in this study was reviewed by the AFRL Internal Review Board (IRB No. FWR20160139N), which determined that this study did not fall under the purview of human use regulations as per 32 CFR 219.109 (f).

The authors would like to acknowledge Study 1 and Study 2 personnel at the Applied Neuroscience Branch (RHCP) for their hard work on the original exercise studies and United States Air Force School of Aerospace Medicine support staff, including medical and laboratory personnel, interns, and exercise physiologists who participated in both original studies. This includes Capt Edward Schlegel and Lt Joshua Bowman who led the exercise groups. Study 1: Dr. Erica Johnson, Dr. Chris Vojta, Mrs. Kyle Traver, and Mr. Chuck Goodyear. Study 2: Dr. Erica Johnson, Dr. Mark Derriso, Mr. Ed Downs, Dr. Adam Strang, Dr. Molly V. Fischer, Maj Steven Rau, PA-C, MPAS, Maj Forrest T. Fornash Jr, PA-C, MPAS, Ms. Molly Wade, Maj Dawn Russell, TSgt Bethany Repp, SSgt Andrew J. Jimenez, SSgt Misty Hobbs, SSgt William Raybon, 2Lt Brian Tabares, Mr. Luke Oaks, Ms. Kylie Fernandez, Mr. Jason Stone, Mr. Jason Eckerle, and MSgt Lisa Thrasher. Thanks also to the Jarvis Gym and Wright Field Fitness Center staff for providing space to conduct Study 2 obstacle course tests. Both original studies were funded by Chief Scientist Seedling Grants to Dr. Edward Eveland, RHCPT.

SUMMARY

Exercise has been associated with cognitive (e.g., attention and memory) and metabolic (VO_2Max) upregulation. Differential effects of exercise type have been seen. High intensity interval training has been shown to produce better VO_2Max compared to steady state exercise. Better VO_2Max has been associated with better cognitive function. Exercise requiring consistent attention (high cognitive engagement, termed HC) has been shown to produce greater cognitive benefits compared to exercise permitting relaxed attention (low cognitive engagement, termed LC). Air Force warfighters exercise as part of their duties, and can be expected to benefit cognitively and metabolically from this requirement. However, highly variable effects of exercise on cognition have been seen. One mechanism underlying exercise effects on cognition is brain-derived neurotrophic factor (BDNF). BDNF is a pleiotropic gene regulating three key physiological systems required to effect exercise behavior: the brain, metabolism, and all three types of muscle. BDNF exists in two main polymorphisms (alleles) in the US population that affect cognitive capacity and response to exercise. A thorough understanding of these alleles' association with cognitive and metabolic upregulation instantiated by exercise is required to guide development of Airman precision training programs. Thus, the purpose of this study was to investigate BDNF allele correlations with exercise type on VO_2Max and a key biomarker affected by exercise: post-acute exercise bout BDNF serum levels. Two previously conducted exercise studies, which included BDNF post-acute exercise bout serum level and post-training VO_2Max, had small cohort sizes and skewed BDNF allele distributions. These two studies were combined to obtain more robust statistical analysis for BDNF allele interaction with exercise type. Even though allele distribution was still not optimal by exercise type, these preliminary results suggest that BDNF allele does affect post-training VO_2Max and serum levels of post-acute exercise bout BDNF regardless of HC or LC exercise training. Additionally, HC exercise produced better VO_2Max and post-acute exercise bout BDNF serum level regardless of allele. This suggests further study is necessary to fully characterize BDNF allele effects by exercise type on Airman cognitive outcomes to facilitate precision exercise prescription for the purposes of rehabilitation post-injury, and initial and ongoing exercise training.

Key Words:

Functional agility training, physical training, cognitive upregulation, brain-derived neurotrophic factor, BDNF, Val66Val, Val66Met, VO_2Max

1.0 INTRODUCTION

Active duty Air Force (AF) personnel are engaged in jobs such as air traffic control, cyber warfare, maintenance and operation of specialized equipment, and aircraft and combat operations that require top level *cognitive skills*. As AF jobs have shifted into more technically and mentally demanding specialty codes, more emphasis has been placed on optimizing individual cognitive performance in the workplace. There are many ways to increase cognitive function, including targeted skill training and practice using software/gaming, optimized nutrition/sleep, and exercise. Exercise has been shown to augment cognitive capacity, including executive attention, memory, and processing speed [1,2,3,4]. Interestingly, exercise type produces differential effects on cognitive augmentation. Exercise which requires consistent effortful attention (high cognitive engagement-HC) has been associated with improved executive attention performance compared to exercise permitting relaxed attention (low cognitive engagement-LC) [5,6,7,8,9,10]. HC exercise requires constant effortful attention to body positioning in space while executing complex, correctly performed movements. Such exercise includes functional agility, martial arts, soccer, dance, and football. LC means participants may relax attention while performing repetitive, largely automated movement sequences such as treadmill running, push-ups, or working closely with a trainer who does the cognitive work of directing trainee attention to proper form and the next exercise in a sequence [11]. Exercise has also been shown to produce highly variable cognitive effects across age and gender groups [12,13,14,15,16].

Exercise-based cognitive augmentation is thought to be mediated by BDNF [17,18]. BDNF is a neurotrophin protein active not only in the brain, but throughout the body. BDNF plays a role in memory formation, stress resilience, muscle function (skeletal, cardiac, and smooth), lipid metabolism, and mitochondrial function [19,20,21]. A meta-analysis by Suzhany et al., [22] showed that exercise resulted in a significant increase in post-acute exercise bout serum BDNF levels and Stothert et al., [23] demonstrated that this result was not a placebo effect. Anderson-Hanley et al., [9] determined exercise requiring effortful attention was associated with higher levels of resting plasma BDNF and better cognitive performance compared to exercise of similar intensity requiring no consistent attentional effort. Intense interval-style training requiring constant shifts of attention has been associated with larger spikes in BDNF serum concentration post-acute intense exercise bout compared to steady-state intense exercise [24]. Oztasonar [25] showed attention-intensive exercise like Taekwondo or boxing was associated with higher post-acute exercise bout serum BDNF levels compared to sedentary controls or long-distance running, with Taekwondo practitioners outperforming all groups. New evidence also suggests cognitive training by itself can increase BDNF serum levels [26]. Thus, resting and post-acute exercise bout BDNF serum levels may be a reliable biomarker of cognitive augmentation. Well-validated methods for isolating BDNF and assessing its concentrations in human blood at rest and post-acute exercise bout exist, and are routinely utilized by Airman Systems Directorate labs for various studies [27].

The BDNF gene contains a polymorphism occurring in the 5' pro-region of the sequence (196G>A, dbSNP: rs6265) [28] which affects processing and cellular release. Humans or animals coding for the BDNF Val homozygote (Val/Val) have demonstrated better memory, better response to stressful events, and better general attention performance whereas BDNF Met carriers (Met homozygotes, Met/Met or heterozygotes, Val/Met) demonstrate less efficient BDNF trafficking and release, and display poorer episodic memory encodement, poorer general prefrontal lobe (attention) function, and greater vulnerability to depression and stress. Interestingly - but paradoxically - the BDNF Met carrier phenotype displays better response inhibition; a specific component of prefrontal lobe cognitive output [21,29,30]. Met carriers may also experience better physical function recovery post-brain injury event [31]. Importantly, exercise may rescue some brain function negatively affected by the Met BDNF variant, and intense exercise may be more beneficial in physical and cognitive domains for Met homo- or heterozygotes compared to Val homozygotes [14,15]. Met carriers may also be more likely to exercise than Val homozygotes [13]. Therefore, BDNF genotype may significantly contribute to the variability in cognitive augmentation associated with exercise type.

Study Design

To provide preliminary information on the interaction of BDNF variant and exercise type with post-training VO_2Max and post-acute exercise bout BDNF serum level, two previous exercise studies conducted in the Human Performance (STRONG) Lab at the Air Force Research Laboratory were combined. Both studies included cognitive testing as well as BDNF Val/Met variant determination and post-training, post-acute exercise bout serum BDNF quantitation. VO_2Max was selected as a key variable for this combined study because both previous studies used the same testing protocol, and because it is a well-validated proxy for: 1) the capacity of the cardiovascular system to transport oxygen to working tissues [32], and 2) the efficiency of aerobic metabolism during intense or prolonged exercise (more than 2 minutes) [33]. VO_2Max is differentially augmented by exercise type, with high-intensity interval-style training resulting in greater augmentation to VO_2Max compared to steady-state training such as Air Force Physical Training (AFPT) or CrossFit [34,35]. Exercise has also been shown to augment mitochondrial function in muscle [36,37]. Interestingly, BDNF may be necessary for efficient mitochondrial function in skeletal muscle [38]. Also, in prior work, the author [7,8] demonstrated that higher VO_2Max was associated with better performance on a neuropsychological test of complex attention function as well as the P3b event-related potential (ERP). This ERP is extracted from electroencephalgraphic data recorded during cognitive testing. The P3b is an index of brain network function, which means in the [7,8] studies, exercise associated with better VO2Max was also associated with improved cognitive behavior and brain function at the network level.[7,8]

Study 1: A pilot study, completed in 2012, compared the effect of traditional AFPT and CrossFit-like training using a pre- and post-training design. VO_2Max and post-acute exercise

bout BDNF serum levels were key dependent variables. AFPT regimens included linear running, weight lifting, team sports, and calisthenics [39,40]. The CrossFit-like training used was a circuit-style program consisting of repetitive resistance and endurance exercises in which cognitive control was outsourced to trainers supervising the group [11,41]. Both of these exercise types permit LC. Subjects were genotyped for BDNF.

Study 2: Completed in 2014, a second study compared intense interval style, functional agility exercise training combined with explicit cognitive training (working memory and decision-making) (termed FAC henceforth) to traditional AFPT (LC). The FAC training incorporated motions from real world actions (e.g., rappelling, field transport of injured warfighters) into training sequences. Functional exercises were updated each week, necessitating participant effortful attention to adapt to changing functional agility exercise requirements. After initial instruction, trainers intervened only if a participant appeared to be having trouble or was doing an exercise incorrectly. In addition, participants were required to be attentive and respond to variable flashing lights at times during training. In some cases, it required quick observation and planned pattern of response to complete. Thus, the FAC training required HC. Participants were tested pre- and post-training on VO_2Max. Due to the BDNF subject matter expert arriving after the start of the study, only post-training, post-acute exercise bout serum BDNF was collected.

Combined Study. LC subjects from both groups were combined into an LC Group. FAC participants were designated an HC group. Note that the FAC (HC) and AFPT+CrossFit (LC) groups are referred to solely according to our cognitive engagement classification system. As noted above, both study groups had been tested with the same protocol for VO_2Max pre- and post-training. Post training, both study groups were tested for post-acute exercise bout serum BDNF levels using the same analysis protocol. All subjects were genotyped in the original studies. Combining these groups allowed investigation of the effect of BDNF polymorphism on post-training, post-acute exercise bout BDNF serum level and post-training VO_2Max, as well as interaction with exercise type.

Study Hypotheses and Objectives

For the combined study, we hypothesized that the HC group would show higher post-training VO_2Max and higher post-training, post-acute exercise bout BDNF serum levels compared to the LC group. We expected Val homozygotes to demonstrate higher levels of serum BDNF compared to Met carriers, and the Met carriers to show higher post-training VO_2Max compared to Val homozygotes. The main objectives of this study were to assess the interaction of exercise training type (HC compared to LC), to identify the BDNF variants, and to conduct association analyses with exercise type, BDNF serum levels, and VO_2Max in Air Force Airmen.

2.0 MATERIALS AND METHODS

2.1 Human Testing Protocol

All protocols were approved by the AFRL Institutional Review Board, protocols FWR20140084H (Study 1), FWR20110018H (Study 2), and FWR20160139N (combined study). All participants signed the Informed Consent Documentation that included consent for genetic testing.

All participants were active-duty male Airmen between the ages of 18 and 40 recruited at Wright-Patterson Air Force Base. Mean age for participants in both studies and all groups was not statistically different. Ages for combined study groups were HC: M=29.11, SEM=1.14, and LC: and M=29.90. SEM=1.19 (M=mean, SEM=standard error of the mean).

2.2 Facilities

Study 1. Testing was conducted at the Wright Field Fitness Center (WFFC)/Health and Wellness Center or Human Performance Lab at Wright-Patterson Air Force Base, Dayton, Ohio. Training was conducted at Jarvis Gym CrossFit area and the RHCP Human Performance (STRONG) Lab.

Study 2. Pre- and post-training data collection was performed at the 711 HPW/RHCP, STRONG lab facilities (formerly the Human Performance Laboratory) of the Air Force Research Laboratory at Wright-Patterson Air Force Base, Dayton, Ohio. FAC training and training data collection were performed at the 711 HPW/RHCP, STRONG lab facilities (formerly the Human Performance Laboratory) of the Air Force Research Laboratory at Wright-Patterson Air Force Base, Dayton, Ohio, the Wright Field Fitness Center (WFFC) at Wright-Patterson Air Force Base, Dayton, Ohio, and Jarvis Gym.

2.3 Study 1

Participants were administered baseline pre-training physiological and cognitive testing. After baseline testing, participants were matched into one of two training groups: 1) Traditional Air Force Physical Training (T) (n=9) or 2) Non-traditional Cross Fit-like training (NT) (n=9). Matching was constrained such that age (M=33, SEM=0.95) and baseline physiological scores were equated across groups. Both regimens were administered for nine weeks by experienced athletic trainers. Both regimens permitted low cognitive engagement (LC). Twelve participants completed all training and testing (T, n=6; NT, n=6). Training protocols are described below.

CrossFit-Like Training Group. Subjects assigned to the CrossFit-like training group were closely supervised according to the following training protocol:

1) Ten-minute fast pace walking warm-up.
2) Perform the recommended CrossFit training routine of the day for 20 minutes.
3) Depending on subject's physical adaptations to the exercise program, exercise intensity was increased five percent bi-weekly to ensure that a continuous training adaptation occurred.
4) Active cool-down for five minutes to prevent blood pooling in the lower extremities was conducted and varied according to individual preference and could include moderate treadmill walking or light stretching.
5) Static stretching of lower extremity major muscle groups.

Traditional Air Force Training. Subjects assigned to the Traditional Air Force group trained according to the following program:

1) Five-minute warm-up. Walk at moderate pace.
2) Walk, jog, cycle, use cardiovascular training equipment such as elliptical trainers or run on a treadmill or outdoors (i.e., any form of *continuous* aerobic activity) for 20 minutes.
3) Resistance training per AFI 10-248 guidance was provided. Sample training was available.
4) Depending on subject's fitness adaptations to this exercise program, subjects were directed to progressively increase their exercise intensity by five percent bi-weekly to ensure that a continuous training adaptation occurred.
5) Active cool-down for five minutes to prevent blood pooling in the lower extremities was conducted and varied according to individual preference and could include moderate treadmill walking or light stretching.
6) Statically stretch major muscle groups used to perform the prescribed exercises.

2.4 Study 2

Active-duty male Airmen between the ages of 18 and 40 were recruited at Wright-Patterson Air Force Base and assigned to the FAC experimental group. The AFPT control group was recruited and filled during the time the experimental group was being tested and trained. Thirty-one male volunteers completed all baseline pre-and post-training physiological and cognitive testing. Each group completed eight weeks of traditional AFPT (n=9) or FAC (n=22). Training protocols are described below.

Study 2 Training Protocols

The two exercise groups were: 1) functional agility plus explicit cognitive training requiring high cognitive engagement (FAC-HC) and 2) traditional Air Force Physical Training permitting

lower cognitive engagement (AFPT-LC). Each group contained different training regimen components (**Table 1**). AFPT-LC controls were instructed to continue their normal personal and squadron PT sessions for eight weeks, and to keep a detailed log of their exercise type and duration. Traditional AFPT-LC encompasses leisure exercise activities as well as more traditional linear training such as running and weight lifting as reflected in the exercise activities listed in Table 1. FAC-HC participants practiced 45 minute sessions of loosely supervised functional adaptive exercises four days per week for eight weeks. Two unique exercise sequences were learned each week, requiring participants to pay constant attention to make sure they were correctly performing movements they were newly learning during each session. For each session they reviewed the exercise plan for the day and any new exercises. Concentration was required to accomplish these tasks which were new to them. Exercises that included randomly flashing lights set up in different patterns were a challenge. FAC-HC participants also continued with their regular squadron PT, but were instructed to avoid any other form of exercise for the duration of the study.

Table 1. Study 2 Training Regimen. Components identified by Group.

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Functional Adaptive Training - HC	**Traditional Air Force PT - LC**
Sprints	Running
Balance	Resistance (weights)
Resistance (weights, bands)	Agility
Agility	Other
Cognitive	Swimming
FitLights:	Biking
Working memory	Yoga
Decision making	Rock climbing
Divided attention	Walking/hiking
DynaVision D2 Trainer	Squadron PT
Visuo-spatial reaction time	
Motor reaction time	
Squadron PT	

Explicit working memory, divided attention, and decision tasks were included in each FAC-HC session for weeks 2, 3, 4, 5, 7, and 8. These tasks were instantiated with the FitLight Trainer™ (FITLIGHT Sports Corp., Aurora, Ontario, Canada) [42]. This equipment includes a set of eight LED lights that can be programmed to present a wide range of working memory, divided attention, and decision-making plus agility tasks. A small, in-house study by the Active Institute,

Aarhus, Denmark [43] in 2012 demonstrated FitLight training delivers an aerobic workout similar to treadmill tests to exhaustion in highly trained participants. Thus, intense aerobic exercise plus working memory, divided attention, and decision training were delivered simultaneously. The Dynavision™ D2 device (http://dynavisioninternational.com/d2.html) was also integrated into FAC-HC training. The D2 device simultaneously trains cognitive and motor domains [44]. See Figure 1 below for a comparison of study designs.

2.5 Common Pre- and Post-training Testing Protocols

Both Studies. VO_2 Max Maximal Treadmill Testing - Modified Bruce Protocol. Pre- and post-training, each subject's maximal VO_2 was determined using the modified Bruce protocol [45] on a Woodway treadmill (Woodway USA, Waukesha, WI). A TruOne 2400 metabolic cart, using open circuit spirometry, was used to measure maximal aerobic fitness level. Participants wore a chest strap slightly below the xyphoid process of the sternum for heart rate monitoring during warm-up, testing, and recovery. Subject exercise heart rates were captured by the software in the metabolic cart throughout the VO_2 testing. Subjects began straddling the treadmill. After a one-minute holding period to verify transmitter communication and allow subjects to adjust to the movement, participants step on and begin the test. Speed and grade were increased every 3 minutes beginning with a 10% grade. The increases continued according to Table 2 below, until participants reached volitional fatigue. Time to volitional fatigue indicated the TTE score (in seconds) and ended the test. The treadmill's speed slowed at the end to induce active recovery until the subject's heart rate dropped below 120 bpm and the test was complete.

Table 2. Modified Bruce Maximal Treadmill Test Stages.

Stage	SPEED	INCLINE
1 (3 –min s)	1.7	10%
2	2.5	12%
3	3.4	14%
4	4.2	16%
5	5.0	18%
6	5.5	20%
7	6.0	22%
8	2.5	12% (recovery)

Study 1. A full-face mask, which covered the nose and mouth, was connected to the breathing valve, otherwise the same procedure as described above.

Study 2. Both of the subject's nostrils were closed off by an external nose clip, and a one-way respiratory breathing valve connected to headgear fastened securely around the head. The nose

clip prevented inhalation and exhalation of air through the nose. The rubber flanges of the mouthpiece connected to the breathing valve prevented leakage of air around the subject's mouth [46], otherwise the procedure as described above.

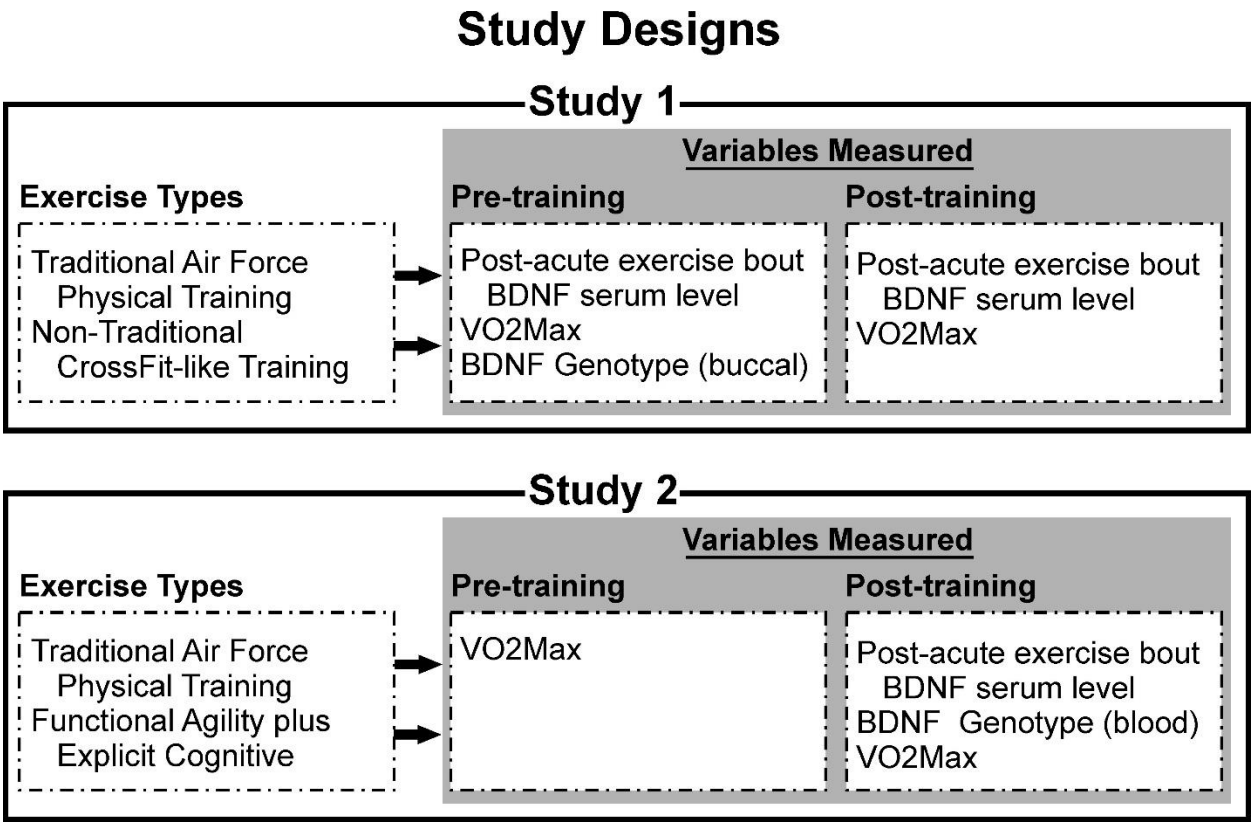

Figure 1. Comparison of Study Designs.

2.6 Post Testing Protocols

2.6.1 Sample Collection

Study 1. Whole blood was sampled through venipuncture from 12 Airmen participants (*n*=6 control group, *n*=6 experimental group) who consented to genetic testing. Buccal cell swabs (3 total) from the inside of the cheek were taken from volunteer subjects and de-identified at point of collection by RHCP personnel. De-identified samples were sent to 711 HPW/RHDJ for BDNF polymorphism analysis.

Study 2. Whole blood was collected post-acute exercise bout from 29 Airmen participants (*n*=8 control group, *n*=21 experimental group) who consented to genetic testing. Samples were de-identified at point of collection, and de-identified samples were sent to 711 HPW/RHDJ for analysis.

2.6.2 Serum Preparation. For both studies, three ml of blood were collected into a serum separator tube (SST) and allowed to clot for 30 min at room temperature. Tubes were centrifuged 15 min at 1000 x g. Serum was removed, separated into 200 µl aliquots, and stored at -20 ºC.

2.6.3 Isolation of DNA from Buccal Swabs. In Study 1, genomic DNA was purified from buccal swabs (Whatman, Cat. No. WB10-0004) using a ChargeSwitch® gDNA Buccal Cell Kit (Invitrogen, Cat. No. CS11021). Each buccal cell sample was transferred to a microcentrifuge tube containing 1 mL of Lysis Mix (prepared by combining 1 mL of ChargeSwitch® Lysis Buffer and 10 µL of Proteinase K). The sample was incubated at 37 °C for 20 min. The digested supernatant was transferred to a new microcentrifuge tube. 40 µL of ChargeSwitch® Magnetic Beads and 100 µL of ChargeSwitch® Purification Buffer were added to the sample, mixed and incubated at room temperature (RT) for 1 min. The sample was placed in a MagnaRack (Invitrogen, Cat. No. CS15000) until the beads formed a tight pellet. The supernatant was carefully removed and discarded, and the pellet was washed twice with 1 mL of ChargeSwitch® Wash Buffer. The tube containing the pellet was removed from the MagnaRack and 150 µL of ChargeSwitch® Elution Buffer were added to resuspend the beads. After a 1 min incubation at RT, the sample was placed back in the MagnaRack until the beads formed a tight pellet. The supernatant containing the DNA was removed and quantitated using a NanoDrop ND-1000 spectrophotometer. Samples were stored at -20 °C.

2.6.4 Isolation of DNA from Whole Blood. In study 2, DNA was isolated from whole blood collected in BD Vacutainer tubes using a QIAamp DNA Blood Mini Kit (Qiagen, Cat. No. 51105). Briefly, 200 µL of whole blood were added to 20 µL of protease and 200 µL of Buffer AL. The sample was vortexed and incubated at 56 °C for 10 min. 200 uL of 100% ethanol were added and the sample was vortexed. The mixture was applied to a QIAamp Mini Spin column and centrifuged at 6,000 x g for 1 min. The column was washed with 500 µL Buffer AWI and centrifuged at 6,000 x g for 1 min. The column was washed with 500 uL Buffer AW2 and centrifuged at 6,000 x g for 1 min. DNA was eluted using 50 µL of Buffer AE and centrifugation at 6,000 x g for 1 min. DNA was quantitated using a NanoDrop ND-1000 spectrophotometer. An A260/280 ratio > 1.8 was accepted as pure.

2.6.5 Identification of BDNF Val66Met Polymorphism

PCR Amplification

A total of 50 ng of DNA (obtained from buccal swabs in study 1 and whole blood in study 2) was used in PCR for amplification of the single nucleotide polymorphism (SNP) containing region. The BDNF (rs6265) polymorphism was genotyped by amplifying a 659 bp fragment using 0.01 nmol/uL of primers:

Forward Primer 5’ – CAC ATG GCA TCC CGG TGA AAG AAA – 3’
Reverse Primer 5’ – AAC CCA TGG GAT TGC ACT TGG TCT – 3’

The PCR was performed by pre-denaturation at 94 °C for 2 minutes, followed by 30 cycles of denaturing at 94 °C for 60 s, annealing at 60 °C for 60 s, and extension at 68 °C for 45 s. Final elongation was performed at 68 °C for 10 min.

Allele Identification by Restriction Fragment Length Polymorphism (RFLP)
The resultant PCR product was genotyped by RFLP by digesting the amplicons with Pml1 (New England Biolabs, Beverly, MA) for 1 hr at 37 °C. The digests were run on a 3% agarose gel in 1X TBE for approximately 2 hrs at 35-70 mAmp. Digests were visualized via UV transillumination. RFLP yielded a 659 bp fragment in the presence of the “A” allele (mut which encodes Met), and 283 and 376 bp fragments in the presence of the “G” allele (wt which encodes Val).

2.6.6 BDNF Serum Concentration. BDNF levels were assayed in duplicate from serum samples using Human BDNF Quantikine ELISA kits (R&D Systems, Cat. No. DBD00). Assays were performed as described in the kit protocol. Briefly, 100 uL of Assay Diluent RD1S were added to each well of a microplate coated with monoclonal antibody specific for human BDNF. 50 uL of standards, controls, or serum samples (diluted 1:30 in Diluent RD6P for study 1 and 1:20 in Diluent RD6P for study 2) were added per well. The plate was incubated for 2 hrs at room temperature (RT). 100 uL of human BDNF conjugate were added to each well, and the plate was incubated for 1 hr at RT. Wells were washed with 3 x 400 uL 1X wash buffer using an automated plate washer (Bio-Tek ELx450, Winooski, VT). Wells were incubated with 200 uL of substrate solution for 30 min at RT in the dark. 50 uL of stop solution were added and absorbance was read at 450 nm using a SpectraMax 190 (Study 1) or SpectraMax M2e (Study 2) microplate reader (Molecular Devices, Sunnyvale, CA).

2.6.7 Study 2 Post-training Obstacle Course. A 13-component obstacle course was prepared to test how training might affect how they accomplish tasks of a functional nature, representative of challenges that might be encountered in mission scenarios. The obstacle course was administered to Study 2 participants post-training. Eleven components were physical challenges. Two components were unpracticed cognitive tests of working memory and cognitively-guided fine motor skill. The cognitive tests occurred at the end of the obstacle course challenge when subjects were within a high-intensity exercise state.

2.7 Statistical Analyses

Alpha was set at 0.05 for all tests. A multivariate analysis of variance (MANOVA) was performed with Group as IV and BDNF serum concentration (pg/mLx1000), and post-training VO_2Max as DVs. Levene's test was run to check for equality of error variances. Box's test was run to check for equality of covariance matrices. Effect sizes are reported as partial eta-squared. Groups were coded as 0 for LC and 1 for the HC groups. This coding was in line with our expectation that the HC group would outperform the LC group on post-training, post-acute exercise bout BDNF serum level. BDNF allele was coded 0 for Met carriers and 1 for Val homozygotes, in line with our hypothesis that Val homozygotes would outperform Met carriers on post-training, post-acute exercise bout BDNF serum level, but a negative correlation would provide evidence in favor of our hypothesis that Met carriers would out-perform Val homozygotes on post-training VO_2Max.

One-tailed Pearson's correlations were performed with Group, post-training, post-acute exercise bout BDNF serum level, BDNF allele, and VO_2Max as variables. Logistic regression, consistent with [47], was considered in an iterative leave-one-out cross-validation strategy, consistent with [48], to determine the underlying ability of our measures to predict group membership. Confidence intervals of a proportion, consistent with [48], were used to provide a margin of error around averaged results from the iterative logistic regression. MATLAB R2014a (Mathworks) and SPSS version 23 (IBM) were used to perform all statistical analyses.

3.0 RESULTS

3.1 Post test Data

Study 1. Both exercise training groups were the same on pre- and post-training VO_2Max and post-training, post-acute exercise bout BDNF serum level. All subjects were Met carriers, including the subjects who dropped out of the study.

Study 2. Four FAC-HC subjects were excluded due to medical issues unrelated to the experiment. Ten FAC-HC trainees and four AFPT-LC trainees dropped out due to personal or occupational circumstances. Thirty-one male volunteers were administered baseline pre-training physiological and cognitive testing, then completed eight weeks of traditional AFPT-LC (n=9) or FAC-HC (n=22). Twenty-eight participants were Val homozygotes, three were Met carriers. Both groups were similar on pre-training VO_2Max. Groups differed on post-training VO_2Max, with the FAC-HC group out-peforming the AFPT-LC group. Groups were similar on post-training, post-acute BDNF serum levels. FAC-HC and AFPT-LC groups performed similarly on the physical components of the obstacle course test. FAC-HC trainees were significantly faster than AFPT-LC controls on the cognitive portion of the obstacle course test in seconds (FAC-HC: 44.85±18.84, AFPT-LC: 69.26±22.83, $p < 0.05$). Thus, after eight weeks, explicit cognitive

training seemed to transfer to unpracticed cognitive tasks during an intense exercise test as practiced by the FAC-HC group.

3.2 Combined Study

Levene's and Box's statistics indicated our data was suitable for the MANOVA procedure. Our overall MANOVA was significant (Wilk's lambda (Λ) ($F(2, 40) = 1134.518$, $p < .001$, *partial eta square* = .983). This result indicates our model explains 98% of the variance in our outcome measures. Group was significant (Wilk's lambda (Λ) ($F(2, 40) = 4.705$, $p = .015$, *partial eta square* = .19). This value indicates 19% of the variance is explained by training type in our outcome measures.

3.3 Statistical Analyses

Group Effect. Group had a significant effect on 1) post-training, post-acute exercise bout BDNF serum level ($F(1, 41) = 4.921$, $p < .032$, *partial eta square* = .107), and post-training VO_2Max ($F(1, 41) = 5.001$, $p < .031$, *partial eta square* = .109). Tukey's honestly significant difference (HSD) showed the experimental group outperformed controls on both measures. The Group comparison of BDNF serum levels is presented in **Figure 1**. Group performance on VO_2Max is presented in **Figure 2**. Descriptive statistics are presented in **Table 3**. Allele carriers by exercise group are presented in **Table 4**. Note that while there were relatively equal numbers of Val homozygotes and Met carriers in the LC group, there were only three Met carriers in the HC group.

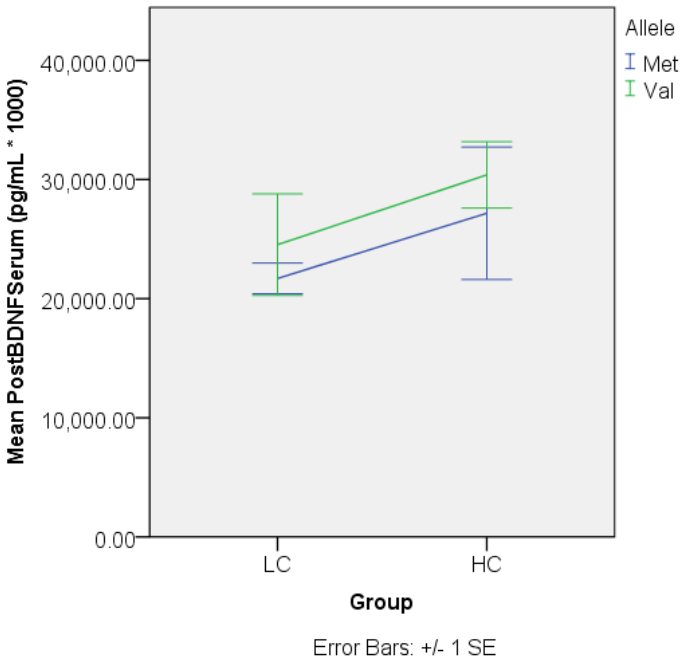

Figure 2. Val homozygotes in both groups showed greater post-training, post-acute exercise bout serum BDNF levels with Val homozygotes significantly outperforming Met carriers for both exercise types (p=0.032)(Table 3 & 4). Serum BDNF is not differentiated by allele.

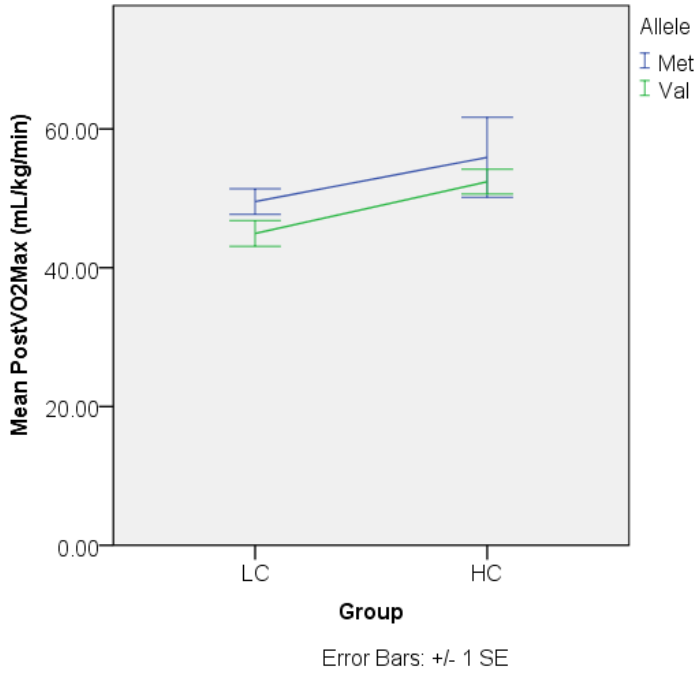

Figure 3. Met carriers in both groups showed greater post-training, post-acute exercise bout VO2Max with Met carriers significantly out-performing Val homozygotes (p=0.031) (Table 3 & 4). VO_2Max is not differentiated by allele.

Table 3. Descriptive statistics by cognitive engagement exercise group.

Descriptive Statistics

Measure	Exercise Group	Mean	SD	N
PostVO_2Max	LC	47.9448	6.39464	21
	HC	52.8768	7.94286	22
Post BDNF in Serum	LC	22911.1943	8856.56722	21
	HC	29946.7909	11674.20949	22

Table 4. Number of allele variants in each exercise group.
LC = Low cognitive engagement HC = High cognitive engagement

	Cognitive Engagement		
Allele	*LC*	*HC*	*n*
Val	9	19	28
Met	12	3	15
n	21	22	43

One-tailed Pearson's Correlations. One-tailed Pearson's correlations are presented in **Table 5**. Group was significantly and positively correlated with BDNF Allele (r=0.456, $p < 0.001$) indicating more Val homozygotes in the HC group (**Table 4**), BDNF serum level (r=0.327, p = 0.02) indicating more BDNF in HC participant serum, and VO_2Max (r=0.330, p = 0.02) indicating higher VO_2Max in HC participants. BDNF was trending to positive correlation with Allele (r=0.253, p = 0.051), indicating Val homozygotes showed higher serum BDNF.

Table 5. One-tailed Pearson's correlations. BDNF=post-training, post-acute exercise bout serum level. VO_2Max=post-training value. *p≤0.10, **p≤0.05, ***p≤0.01

	Group	Allele	BDNF
Allele	***0.456		
BDNF	**.0.327	*0.253	
VO_2Max	**0.361	-0.056	0.150

Backward Logistic Regression. A backward multiple logistic regression was considered with iterative training and testing via a comprehensive leave-one-out cross-validation. The leave-one-out cross-validation was performed as follows: first one observation from each group was segregated to form a testing set with $N_1 = 1$and $N_0 = 1$. The remaining data ($N_1 = 17$ and $N_0 = 19$) was used to train a logistic regression model. Accuracy and significance of features was then considered. This process was repeated for all possible combinations (360) of training conditions and testing observations.

Prior to feature selection via backward selection, the training and testing classification accuracy across all iterations was computed and is presented in **Figure 4**. The prior probabilities of the training set are 0.47 for group 1 and 0.53 for group 2 and the prior probabilities for the testing set are 0.50. Thus, with the training accuracies in **Figure 4** being consistently between 75% and 89%, mean of 80.3% ±2.0%, and the testing accuracy fluctuating between 0 and 100%, with a mean of 74.7% ±2.3%, indicates that, overall, the two groups are effectively discriminated since the results are consistent above the prior probabilities in a random guess.

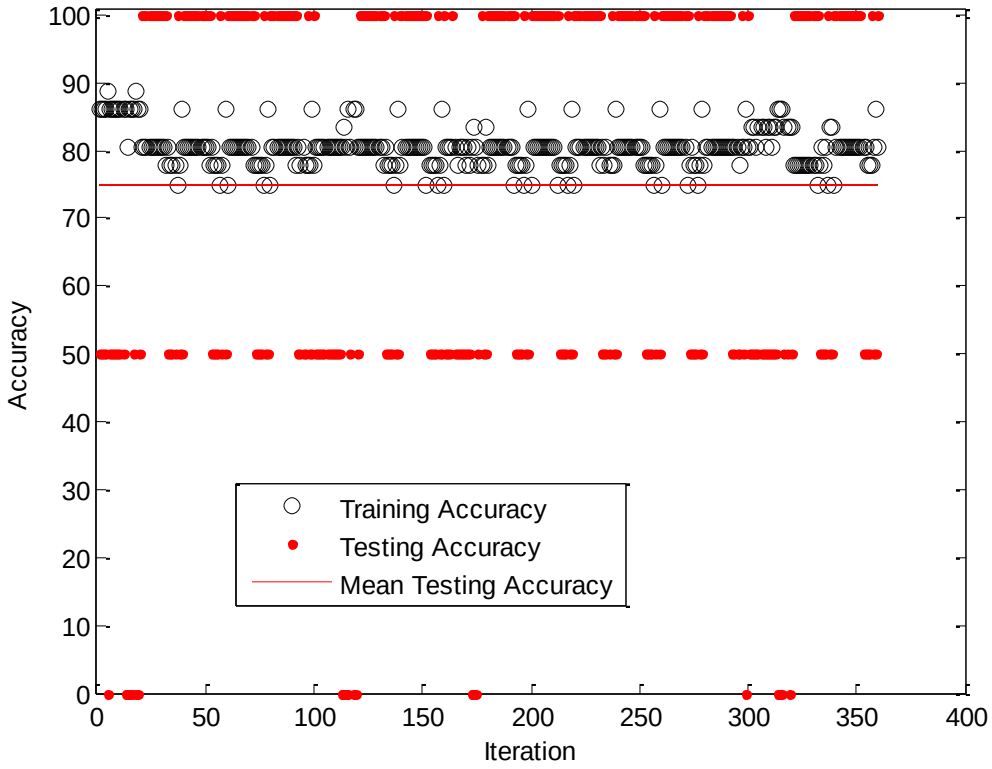

Figure 3. Training and testing classification accuracy across iterations for the full dataset.

Consistency is seen across the iterations in logistic regression coefficients for Serum, VO_2 and Age, **Figure 5**. Additionally, *p*-values are seen as largely significant for VO_2, and Allele across iterations; however, the p-values are largely between 0.1 and 0.2 with age being consistently not significant across iterations. Thus feature selection was considered whereby insignificant features (based on the mean p-value across iterations) were discarded and new logistic regression models were created. Complete logistic regression results are presented in Appendix I.

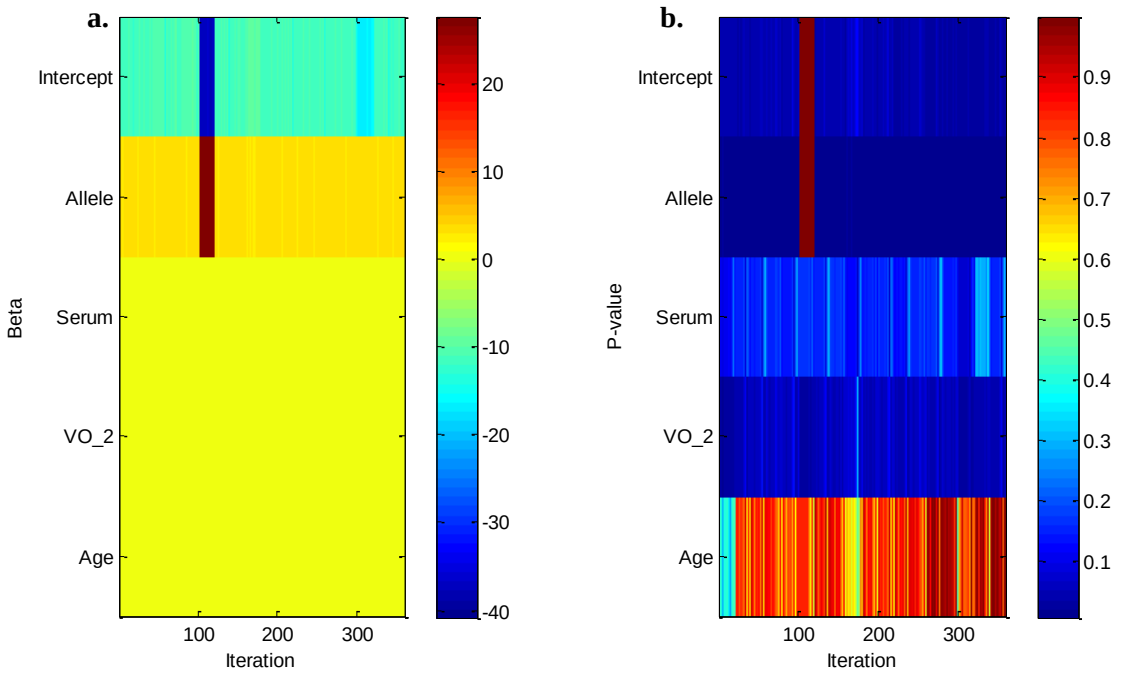

Figure 4. Heat Map Indicating Logistic Regression Coefficients (a) and p-values (b) for each data feature, across iterations.

4.0 DISCUSSION

Augmenting Airman cognitive capacity is a key focus of the Airman Systems Directorate [48]. Exercise has been associated with cognitive upregulation including memory and effortful attention are vital cognitive skills for many, if not all, Airman tasks. Airmen routinely exercise as a part of their jobs, but while consistent exercise training maintains a level of physical capacity, its effect on cognitive upregulation is highly variable [12,16]. A key mechanism contributing to variance underlying exercise upregulation of cognitive capacity has been identified: the pleiotrophic gene, brain-derived neurotrophic factor (BDNF) [4,9,17,18]. Direct association of BDNF biomarkers (post-acute exercise bout serum or plasma levels) and cognitive capacity are scarce, likely due to the fact that BDNF has a complex regulatory role in three key systems required for exercise: 1) brain [50,51,52,53,54], 2) metabolic [52,55,56,57], and 3) muscular (skeletal [58], cardiac [59], and airway smooth [60]). Common and different molecular pathway mechanisms of BDNF in these key organ systems are still being elucidated. Key pathways for mitochondrial biogenesis in brain and muscle may be similar [53-62]. Thus, it is possible indirect interactions between system-wide BDNF-mediated responses to exercise stimuli underlie cognitive upregulation that does not show up in standard inferential statistical procedures seeking direct significance. However, one BDNF biomarker has been directly associated with cognitive capacity as well as BDNF serum levels and BDNF genotype.

Published studies indicate that Val homozygotes have greater post-acute exercise bout BDNF serum levels compared to Met carriers [63] as well as better cognitive function [28]. But, Met carriers have shown greater propensity to exercise [13] and have been shown to receive greater benefits from intense exercise compared to Val homozygotes [15]. Exercise seems to ameliorate some of the cognitive deficits associated with BDNF Met variants [14], so investigating the effect of variant on BDNF transcript production in response to exercise stimuli is critical. Thus, two recent exercise training studies from the Human Performance (STRONG) Lab in the Applied Neuroscience Branch of the Airman Systems Directorate, which included BDNF variables were combined to investigate the effects of BDNF variant and exercise type on post-training VO_2Max and post-training, post-acute exercise bout serum BDNF levels.

Study 1 intended to test collected samples to analyze serum BDNF and to investigate the differential effects of traditional AFPT and non-traditional CrossFit-like exercise on resting and post-acute exercise bout BDNF serum levels. Acute exercise was instantiated with the maximal treadmill VO_2Max protocol described in Methods Section above. Study 1 participants showed no pre- or post-training difference by group on resting or post-acute exercise bout BDNF serum levels or post-acute exercise bout VO_2Max. This study finding was likely due to the close similarity between exercise regimens as both were considered intense. Unexpectedly, all subjects in Study 1 were Met carriers, well outside the normal BDNF allele distribution seen in the US: 70-80% Val homozygotes and 30-20% Met carriers [64] and was an unexpected allele distribution. Additionally, this study did include cognitive variables that were determined not sensitive enough to reveal pre- and post-training effects.

The Study 2 design did not originally include any BDNF variables. It was designed to document the effects of traditional AFPT and a novel functional adaptive plus explicit cognitive training (FAC) exercise regimen on performance in an obstacle course with practiced physical challenges and unpracticed cognitive components. Due to contracting difficulties, the BDNF/exercise subject matter expert (SME) originally intended to work on Study 1, did not arrive at AFRL until after the start of Study 2 pre-testing. Since the BDNF SME's National Research Council Post-doctoral project was contracted to examine exercise effects on BDNF serum levels [65], after much discussion, and consult with a genetics SME in RHDJ, it was decided to collect BDNF genotype and post-training, post-acute exercise bout serum BDNF on all Study 2 subjects. BDNF allele distribution in Study 2 was 28 Val homozygotes and 3 Met carriers. Again, this distribution was well outside the normal BDNF allele distribution seen in the US, but in the opposite direction compared to Study 1. This study did include cognitive variables that were also not sensitive enough to reveal pre- post-training effects.

Sample Size and Allele Distribution Limitations and Solution

The two studies discussed in this report each included BDNF allele, post-acute exercise bout BDNF serum level, two different exercise types (HC and LC), and post-training VO_2Max.

However, the first study (Study 1) suffered from a small sample size and similar exercise regimens that both permitted low cognitive engagement (LC), and statistically similar training outcomes (serum BDNF and VO_2Max). In addition, genetic analyses of the Val/Met polymorphism (rs6265) indicated that all participants were Met carriers. The second study (Study 2, with FAC-HC and AFPT-LC groups) also suffered from a small sample size (31 participants) and contained 28 Val homozygotes, but only three Met carriers. Recall, Val carriers could be expected to show better memory performance compared to Met carriers. Thus, any group with a high percentage of Val carriers could be expected to outperform any group with a high percentage of Met carriers on memory tests.

To address the problematic issues of low sample size and the limitation in Val/Met allele genetic distribution across groups, these two studies were combined. AFPT controls from both studies and CrossFit-like participants from Study 1 were combined into an LC group. FAC participants from Study 2 were defined as an HC group. A backward logistic regression suggested that the decision to combine Study 1 LC with Study 2 LC participants and compare them to Study 2 HC participants was justified and robust (3.3.1) (see **Figure 4 and 5** in the **Appendix**).

Exercise Effects

For the combined study, exercise type was evenly distributed in the sample, with 21 LC and 22 HC participants. We had hypothesized the HC group would show significantly higher post-acute exercise bout BDNF serum levels [17,22, 24, 25] and post-training VO_2Max [33] compared to the LC group. That is what we found (see **Figures 2 and 3** respectively, and **Table 3**). Since we had balanced exercise type sample sizes, and these results are in line with those reported in the literature described, they may be considered robust. Further, correlations support this interpretation. Recall, the groups were coded so correlations were easily and intuitively interpretable: HC was coded 1 and LC, 0, which was in line with our prediction that HC participants would outscore LC participants. So, the group was positively and significantly associated with post-training, post-acute exercise bout serum BDNF level and post-training VO_2Max (see **Table 5**), and membership in the group coded 1 (HC) was associated with better scores on our key outcome variables previously mentioned.

BNDF Allele Effects

BDNF allele distribution for the combined studies was 28 Val homozygotes and 15 Met carriers. This sampling condition is considered an unbalanced sample size [65], which can affect reliability of MANOVA results. Again, Val carriers could be expected to show better memory performance compared to Met carriers. Thus, any group with a high percentage of Val carriers could be expected to outperform any group with a high percentage of Met carriers on memory tests. Future studies with serum BDNF and neuropsychological tests of memory function should take this into account if unequal allele distributions are seen in test groups. Critically, the alleles were not well distributed between exercise types. There were only three Met carriers in the HC

group (see Table 4). These two facts affect the ability of this study to robustly assess allele interaction with exercise type on post-training, post-acute exercise bout serum BDNF level and post-training VO_2Max. Our original hypothesis stated we expected Val homozygotes to show higher levels of serum BDNF compared to Met carriers [27], though some literature suggested Met carriers could be expected to respond well to intense exercise in the physical [13,21,31] and cognitive [29,30] domains. As mentioned above and is discussed in more detail below, neither original study had sensitive measures of cognitive capacity, but we did have VO_2Max, a gold standard proxy for cardiovascular and metabolic capacity [32,33]. Because of work by Hooper et al. [13], we had predicted Met carriers would show higher VO_2Max compared to Val homozygotes. That trend is what we observed for both exercise types (see **Figure 3**). While not statistically significant, the data also showed Val homozygotes showed a tendency for higher post-training post-acute exercise bout serum BDNF level compared to Met carriers regardless of exercise type (see **Figure 2**). Finally, allele status did significantly and positively correlate with post-acute exercise bout serum BDNF levels (see **Table 5**). Recall, alleles were coded Val, 1 and Met carrier, 0. This result demonstrated that the Val allele was associated with higher serum BDNF levels for both exercise types. However, it is probable that the skewed distribution of Met carriers in the HC group affected the study results to allow clear demonstration of the true nature of allele interaction with exercise type.

Follow-on Study Design Modifications

One of the intents of both original studies described here was to examine associations between exercise type, BDNF serum level, VO_2Max and cognitive performance. However, as mentioned above the cognitive tests used in each study showed strong ceiling effects. This means that all participant scores were at the top of the scoring range at pre-test, such that there was little room for improvement post-training. AFRL labs have been searching for cognitive tests difficult enough to show training effects between Airman experimental groups. Such tests would be sufficiently difficult and produce mid-range scores at pre-test, providing significant room for training effect enhancements in terms of score improvement. A 'Distribution D' study in the STRONG Lab in the summer of 2015 demonstrated such tests are possible. Further work to develop a sensitive and specific cognitive test bank for AFRL labs is in the planning stage. Additionally, Study 1 exercise regimens were too similar to produce differential training effects, and Study 2 logistics prevented obtaining pre-training BDNF serum levels for the AFPT experimental group. Follow-on studies will include pre- as well as post-training BDNF assays, clearly formulated HC and LC exercise regimens, and sensitive and specific cognitive tests of memory, effortful attention, and decision-making. Val/Met pretesting is likely necessary to insure equal allele distribution between exercise groups. The described modifications in a follow-on study design should help provide robust results. However, there are methodological issues with respect to BDNF quantitation related to sources of circulating BDNF protein (see below). The effects on circulating BDNF levels derived from these different sources must be disentangled.

Differential Results of BDNF Assay Type

Circulating BDNF can be assayed in serum [22, 24, 25, 26] or plasma [9, 67, 68, 69]. Studies investigating BDNF serum and plasma levels in young healthy males during exercise and in recovery post-acute exercise bout reported different levels of circulating BDNF protein depending upon assay type [67]. These studies also reported changes in BDNF plasma and serum protein post-intense acute exercise bout which were different than those reported in the literature. However, they did not genotype their subjects, so allele effects may be one contributor to this discrepancy [28]. Importantly, they found greater amounts of BDNF in plasma compared to serum post-acute exercise bout and plasma levels returned to baseline more slowly than serum BDNF levels. This may be due to different sources of circulating BDNF protein in serum and plasma [69,70,72]. The BDNF gene is active in megakaryocytes and its transcripts are stored in platelets before they bud off and enter the circulation. Further evidence shows that platelet reactions to stimuli may contribute to circulating BDNF protein in serum [72]. Further, Rasmussen et al. (2009) showed that since platelets may contribute to serum levels of BDNF protein, plasma may be better for revealing BDNF release from the brain [73]. They also documented BDNF levels in arterial to internal jugular venous difference in plasma for healthy, human males at rest and during intense, long-duration rowing exercise. They found a large increase in circulating plasma BDNF post-exercise. This suggests assays of BDNF in plasma may reflect BDNF release as a result of exercise stimuli without the confounding levels of platelet BDNF load, and could be used in future AFRL BDNF studies.

BDNF miRNAs

It is important to realize that exercise requires three key systems whose function is regulated by BDNF transcripts: 1) brain [50-54], 2) metabolic [52, 55, 56, 57], and 3) muscular (skeletal [58], cardiac [59], and airway smooth [60]). BDNF protein levels are regulated by microRNAs (miRNAs) [74]. MiRNAs are involved in mostly negative, but sometimes positive feedback loops affecting mRNA transcription or effecting mRNA degradation in the central nervous system [75-77]. MiRNAs have been found circulating in the periphery [79] and reliable methods for their assay are available [80]. Critically, miRNAs have been implicated in tissue cross-talk in the periphery [79]. Is it possible BDNF miRNAs effect tissue cross-talk in response to exercise stimuli? This is a question that has not been addressed in the current BDNF miRNA literature. While the high variability seen in exercise effects on BDNF biomarkers such as post-acute exercise bout serum BDNF level, changes across studies can be partially addressed by including BDNF genotype as a mediator in future studies. Finally, the role of BDNF miRNAs in regulation of BDNF in the central nervous system and those in periphery must be investigated.

5.0 CONCLUSION

In spite of these limitations, this study suggests that BDNF allele has differential effects on post-training, post-acute exercise bout serum BDNF and post-training VO_2Max. Further, HC compared to LC training showed significant differential effects on post-training, post-acute exercise bout serum BDNF and post-training VO_2Max. Thus, future studies taking the above concerns into account are warranted. Those results can guide Air Force Trainers in precision training of new recruits and career Airmen, per the sense-assess-augment taxonomy for human effectiveness put forth by the 711 Human Performance Wing.

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7.0 LIST OF SYMBOLS, ABBREVIATIONS, AND ACRONYMS

AFPT	(traditional) Air Force physical training
BD	blood vacutainer tubes
BDNF	brain-derived neurotrophic factor
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immunosorbent assay
FAC	functional adaptive training plus explicit cognitive training
HC	high cognitive engagement
HSD	honestly significant difference
LC	low cognitive engagement
M	arithmetic mean
MANOVA	multivariate analysis of variance
mut	mutation
PCR	polymerase chain reaction
PT	physical training
RFLP	restriction fragment length polymorphism
s	second
SEM	standard error of the mean
SME	subject matter expert
SST	serum separator tube
wt	wild type

APPENDIX A.
COMPLETE BACKWARD LOGISTIC REGRESSION RESULTS

Reconsidering the data without age as a variable in the model yielded **Figure A1**. In **Figure A1**, Training and testing accuracy is seen to have improved when compared to **Figure 4**. While the logistic regression coefficients, **Figure A2a**, and p-values, **Figure A2b**, are seen to be consistent with the original models (**Figure 5**). Feature selection was considered again, with serum excluded from the model, yielded performance seen in **Figure A3**. Training and testing accuracy is seen to have improved. While the logistic regression coefficients, **Figure A4a**, and p-values, **Figure A4b**, are seen to be consistent with the original models.

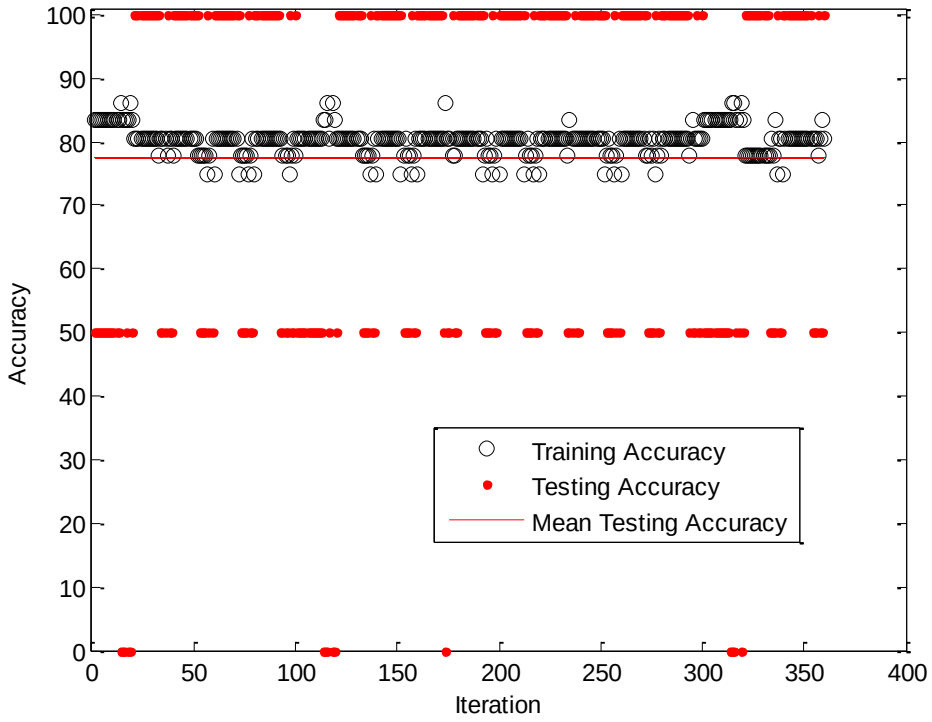

Figure A1. Training and testing classification accuracy across iterations with age excluded.

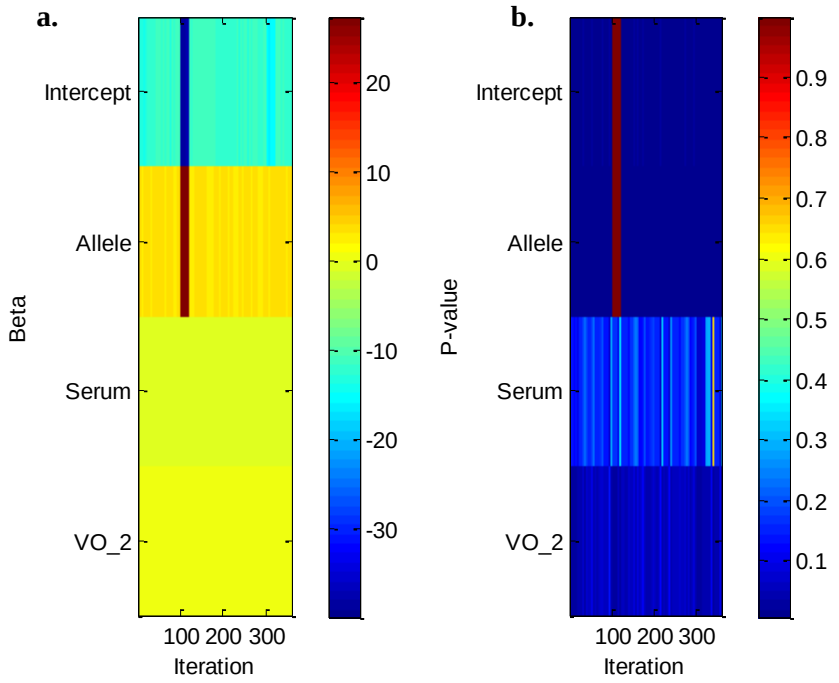

Figure A2. Logistic regression coefficients (a) and p-values (b) for each data feature, across iterations with age excluded.

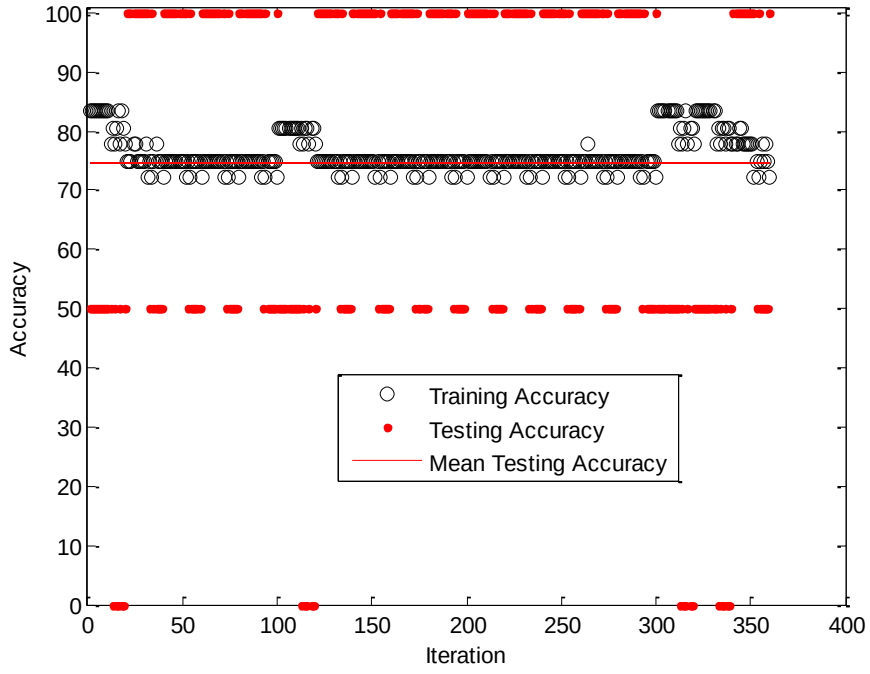

Figure A3. Training and testing classification accuracy across iterations with age and serum excluded.

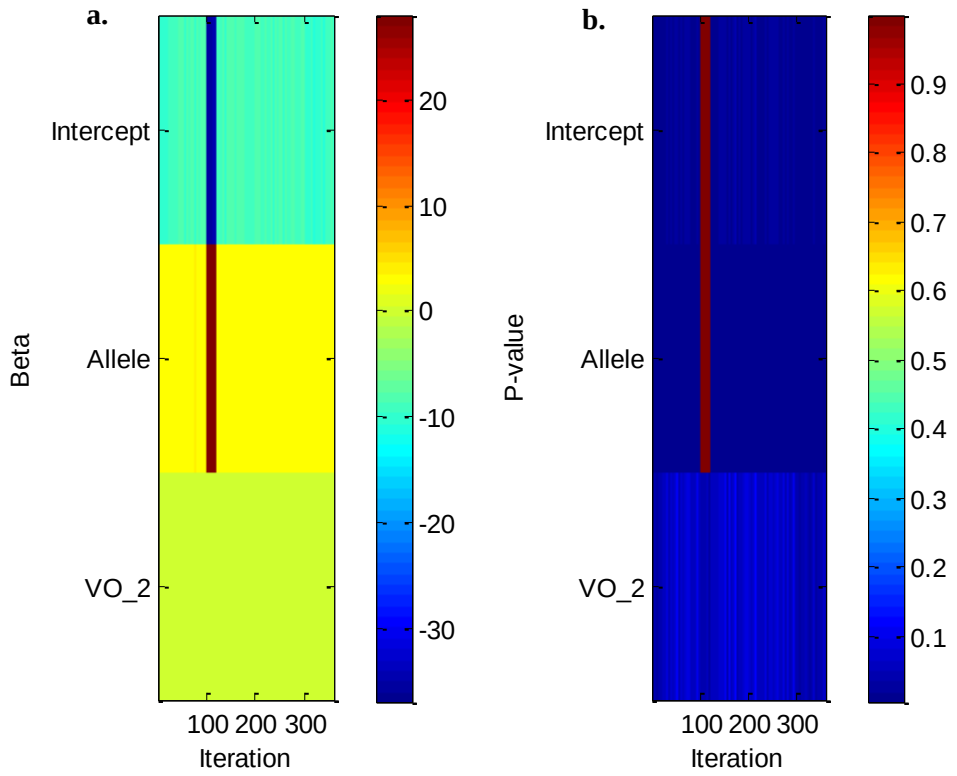

Figure A4. Logistic regression coefficients (a) and p-values (b) for each data feature, across iterations with age and serum excluded.

Feature selection was considered again, with Allele excluded from the model, yielded performance seen in **Figure A5**. Although allele was correlated 0.45 to the class groups, its contribution to accuracy was great. Removing allele and relying on VO_2 resulted in approximately 60% accuracy, while this is not a random chance it is a significant drop from above. However, the logistic regression coefficients, **Figure A6a**, and p-values, **Figure A6b**, are seen to show the predominant statistical significance of VO_2 in discriminating between groups.

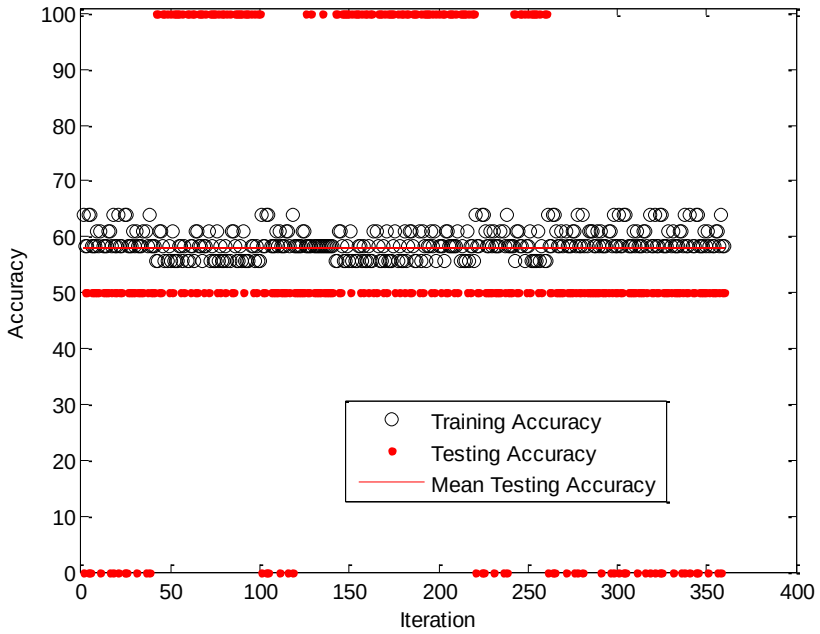

Figure A5. Training and testing classification accuracy across iterations with age, serum and allele excluded.

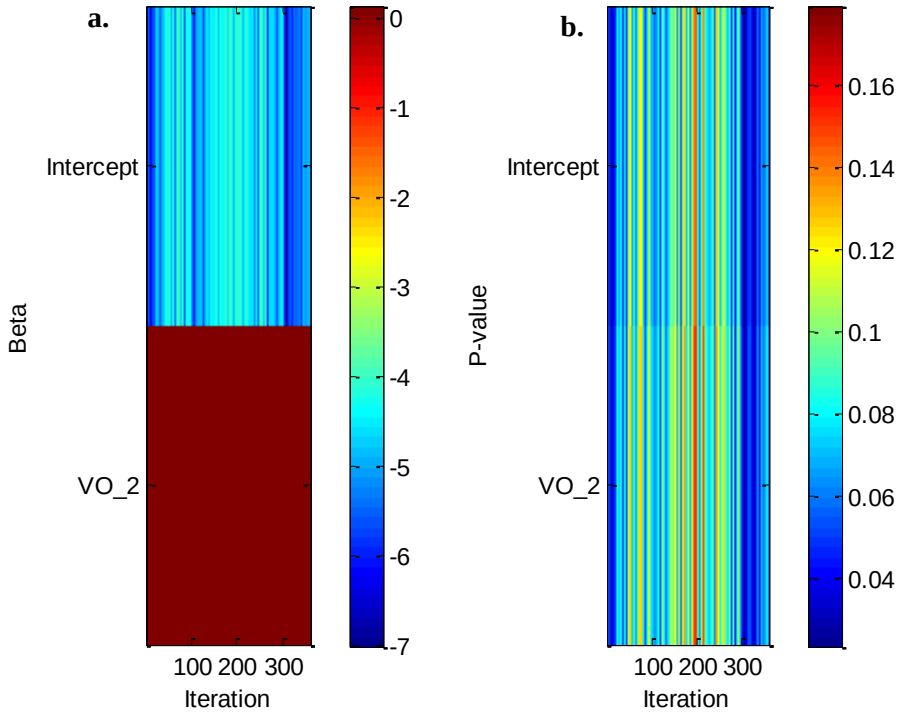

Figure A6. Logistic Regression Coefficients (a) and p-values (b) for each data feature, across iterations with Age, Serum and Allele excluded